Supporting Information

Pfluger *et al.* 10.1073/pnas.0802917105

SI Methods

Generation of Transgenic Mice and Animal Experimentation. A large genomic DNA segment (174 kb) containing the murine Sirt1 gene and cloned into the *B*acterial *A*rtificial *C*hromosome(BAC) vector pBAC3.6 was obtained from CHORI (identification no. RP23-119G23) (http://www.chori.org). The terminal sequences of the insert were confirmed with primers complementary to the insert-flanking elements of the vector, namely, SP6-BAC 5′-atttaggtgacactatagaat-3′ and T7-BAC 5′-taatacgactcactataggg-3′. The integrity of the BAC was further confirmed by digestion with several restriction enzymes and Southern blot analysis (supporting information (SI) Fig. S1). For transgenesis, the Sirt1-containing BAC was digested with PI-SceI, thus linearizing the BAC in the vector at a position adjacent to the T7 end of the genomic insert (the T7 end is upstream of the Sirt1 gene, at the Ctnna3 gene; see Fig. 1*A*). Pronuclei of fertilized oocytes, derived from intercrosses between (C57BL6 × CBA)F_1 mice, were injected with ≈2 pl of a DNA solution containing the linearized BAC. The resulting offspring was analyzed for the presence of the transgene by using PCR reactions that amplify either the vector (primers pBAC-F 5′-agatagttcaccggggtgagaa-3′ and pBAC-R 5′-ttcggtcgaagagtatctggtg-3′), or the T7 insert-vector junction (primers T7-BAC, see above, and T7-tg 5′-agcttcttgcacaccacatggagtt-3′). One founder was identified that overexpressed Sirt1, abbreviated here as Sirt1-tg. The number of integrated BAC copies was determined in two ways: first, quantification of Southern blot analysis against Sirt1 gene indicated the presence of a single transgenic copy in addition to the two endogenous alleles (Fig. S2); and, second, Southern blot analysis against a terminal fragment of the BAC genomic insert indicated the presence of a single transgene–genome junction (data not shown), again supporting the presence of a one transgenic copy. This founder Sirt1 [+/+;tg] mouse was back-crossed for four generations with pure C57BL6 mice; in this manner, all of the mice used in this study share a genetic background that is 97% C57BL6. Mice were housed at the pathogen-free barrier areas of the Spanish National Cancer Research Center (CNIO) and at the University of Cincinnati. Sirt1-heterozygous mice were kindly obtained from Fred Alt, Harvard Medical School.

Mice were fed either with a standard chow diet (Harlan Teklad LM-485), or with a high-fat diet (Research Diets 12451, 45% kJ from fat) starting when the mice were ≈2 months old and for a total of 19 additional weeks. Whole-body composition (fat and lean mass) was measured by using well established quantitative NMR technology (EchoMRI). To assess LPS susceptibility in each genotype, mice were injected i.p. with 20 mg/kg of *Escherichia coli*-derived LPS serotype 0111:B4 (Sigma) and then monitored every 8 h for a 72-h period. All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committees (IACUC) of the University of Cincinnati and the Guidelines for Humane Endpoints for Animals Used in Biomedical Research at the CNIO.

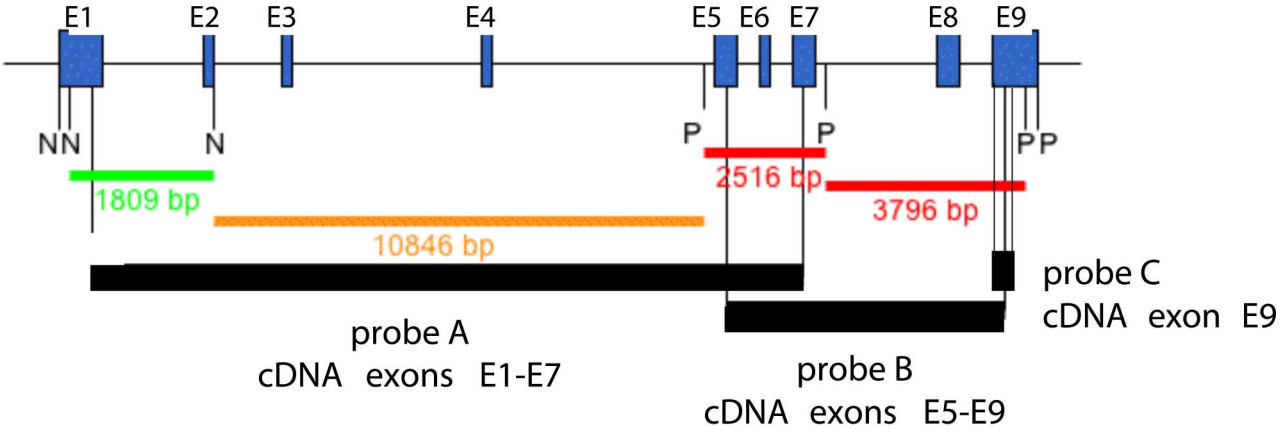
DNA, RNA, and Protein Analyses. Southern and Northern blot analyses were performed by using standard procedures. The probes and primers used to obtain the probes are explained in the corresponding figure legends. Real-time PCRs to validate for candidate genes and for the ribosomal housekeeping gene L-32 were performed on a Bio-Rad iCycler by using iQ SybrGreen Supermix (Bio-Rad). Sequences of primers are listed in (Table S2). Western blot analyses were performed by standard procedures. For detection of Sirt1, we used rabbit polyclonal antibody ab12193 (AbCam), working dilution 1:2,000; and for β-actin, we used monoclonal antibody AC-15 (Sigma), 1:5,000.

Mouse Embryonic Fibroblasts. Luciferase assays were done by using Lipofectamine LTX and PLUS reagent (Invitrogen) for the transfections. We used pNFκBLuc (Clontech) reporter vector and E1fα-Renilla vector as transfection control. TNFα (Promega) was added at 10 ng/ml and luminescence was measured 6 h later by using a Glomax luminometer (Promega).

Blood Parameters. Tail blood was collected 2 h after the onset of the light phase either after an overnight fast, or in ad libitum-fed mice by using EDTA-coated Microvette tubes (Sarstedt), and immediately chilled on ice. Glucose levels were determined from whole blood at the beginning of each blood collection by using the TheraSense Freestyle glucometer. Plasma insulin levels were quantified by using a RIA from Linco (Sensitive Rat Insulin RIA, Linco Research). Nonesterified fatty acid levels (NEFAs) were measured by using an enzymatic assay kit from Wako (NEFA C, Wako). Plasma cholesterol levels were determined by using Infinity Cholesterol reagent, and plasma triglycerides were quantified by using Infinity Triglyceride reagent (Thermo Electron). Plasma leptin and adiponectin levels were measured by using the Quantikine mouse leptin and adiponectin immunoassays, respectively (R&D Systems). All assays were performed according to the manufacturer's instructions.

Metabolic Phenotyping. Metabolic performance (energy intake and energy expenditure) and home-cage activity were studied by using an automated combined indirect calorimetry system (TSE Systems GmbH). Before the experiment, the mice were allowed to acclimatize to the air-tight cages for 16 h, and subsequently oxygen consumption and CO_2 production were measured every 45 min for a total of four light and four dark phases (100 h). The respiratory quotient (RQ; VCO_2/VO_2) and energy expenditure were calculated by using standard in-house software. Simultaneously, intake of food and water was determined continuously by integration of scales into the sealed-cage environment. In parallel, home-cage locomotor activity was determined by using a multidimensional infrared light beam system with beams installed on cage-bottom and cage-top levels and activity being expressed as beam breaks. Stationary motor activity (fidgeting) was defined as consecutive breaks of one single light beam, and ambulatory movement as consecutive breaks of two different light beams.

For the glucose tolerance test (GTT), WT and tg mice on both diets were subjected to 6 h of fasting and injected i.p. with 2 g of glucose per kg of body weight. The insulin tolerance test (ITT) was performed similarly, with an initial fasting for 6 h, and subsequent i.p. injection of 0.75 IU insulin per kg of body weight (Eli Lilly and Co., Humalog Insulin). The pyruvate tolerance test (PTT) was performed by i.p. injection of 2 g of pyruvate per kg of body weight, after an initial 6-h fast. In all tests, tail blood glucose levels were measured with a glucometer (TheraSense Freestyle) at the indicated times after injection.



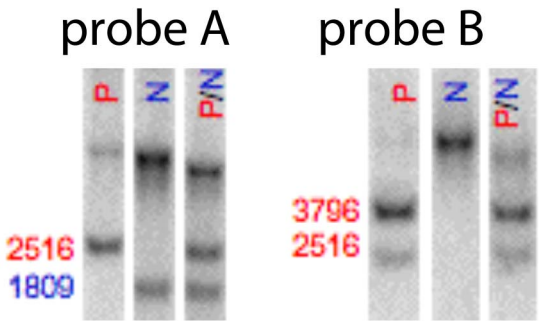


Fig. S1. Integrity of the genomic insert present in the Sirt1 BAC. The Sirt1-BAC DNA prepared from bacteria was digested with restriction enzymes PacI (P) and NotI (N), alone or in combination, and then probed with the indicated probes obtained from Sirt1 cDNA. The results of Southern blot analysis are shown in the bottom part. The probes are derived from Sirt1 cDNA, as indicated in the top scheme, together with the predicted DNA fragments generated by PacI and NotI. The primers used to obtain the probes are listed in Table S2.

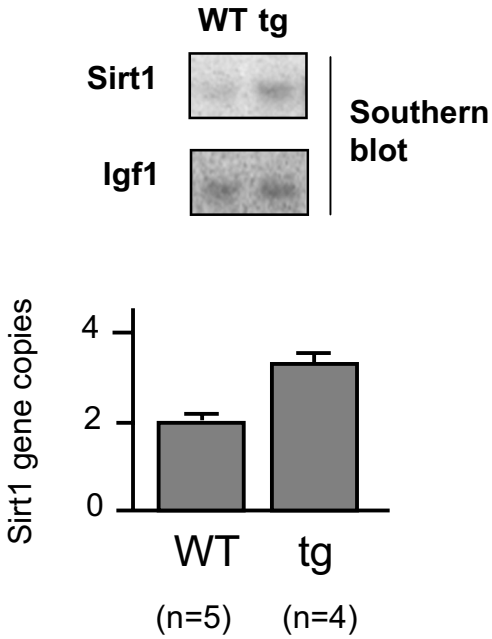
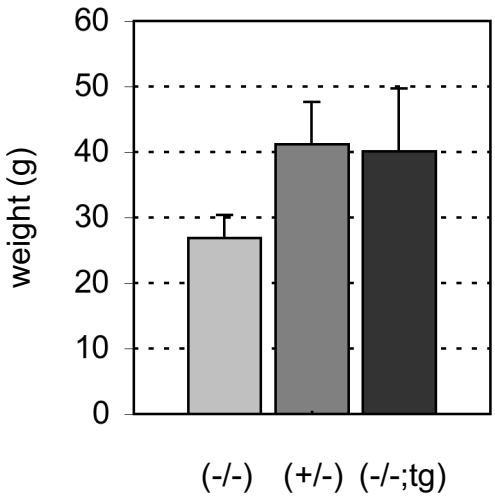


Fig. S2. Gene copy number in Sirt1-tg mice. The number of integrated BAC copies was determined in two ways: first, quantification of Southern blot analysis against Sirt1 gene indicated the presence of a single transgenic copy in addition to the two endogenous alleles (shown in this figure); and, second, Southern blot analysis against a terminal fragment of the BAC genomic insert indicated the presence of a single transgene-genome junction (data not shown), again supporting the presence of one transgenic copy. The Southern blot analysis shown here was performed by using genomic DNA from the tail tips, digested with restriction enzyme EcoRI, and probed with a cDNA fragment of Sirt1 corresponding to exon 9 (see probe C in Fig. S1). To control for DNA loading, membranes were subsequently reprobed with a probe that detects Igf1. (*Upper*) One representative example. (*Lower*) Quantification of wild-type ($n = 5$) and transgenic ($n = 4$) mice. Quantification indicates that transgenic mice have a single additional copy of the Sirt1 gene (WT mice = 2 copies; tg mice = 3 copies).

A

Sirt1(+/-;tg) x Sirt1(+/-) offspring		
Sirt1 genotype	expected Mendelian ratio (%)	observed ratio (%) (n=50)
(+/+)	12.5	16
(+/-)	25	30
(-/-)	12.5	6
(+/+;tg)	12.5	14
(+/-;tg)	25	22
(-/-;tg)	12.5	12

B



C

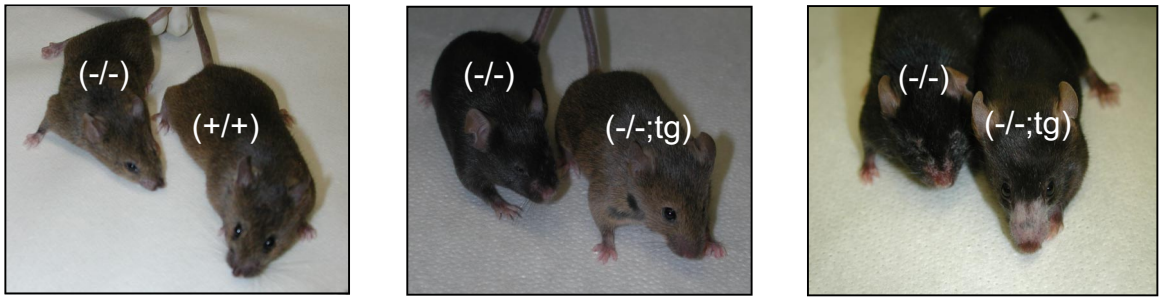


Fig. S3. Genetic rescue of Sirt1-null phenotypes by the Sirt1-tg allele. (*A*) The partially penetrant perinatal lethality of Sirt1 deficiency was rescued by the Sirt1-tg allele. Sirt1(−/−;tg) mice were born with the expected Mendelian ratio, whereas Sirt1(−/−) mice were born at a 50% ratio compared with the expected Mendelian proportion. (*B*) Sirt1(−/−;tg) mice had a body weight similar to Sirt1(+/−) mice (all mice of 8–12 months of age), which is in agreement with the fact that both genotypes carry a single functional copy of the Sirt1 gene. In contrast, viable Sirt1(−/−) mice had a lower body weight. (*C*) Sirt1(−/−) mice have noticeably small eyes (microphtalmia), whereas Sirt1(−/−;tg) mice have normally sized eyes, comparable to Sirt1(+/+) controls.

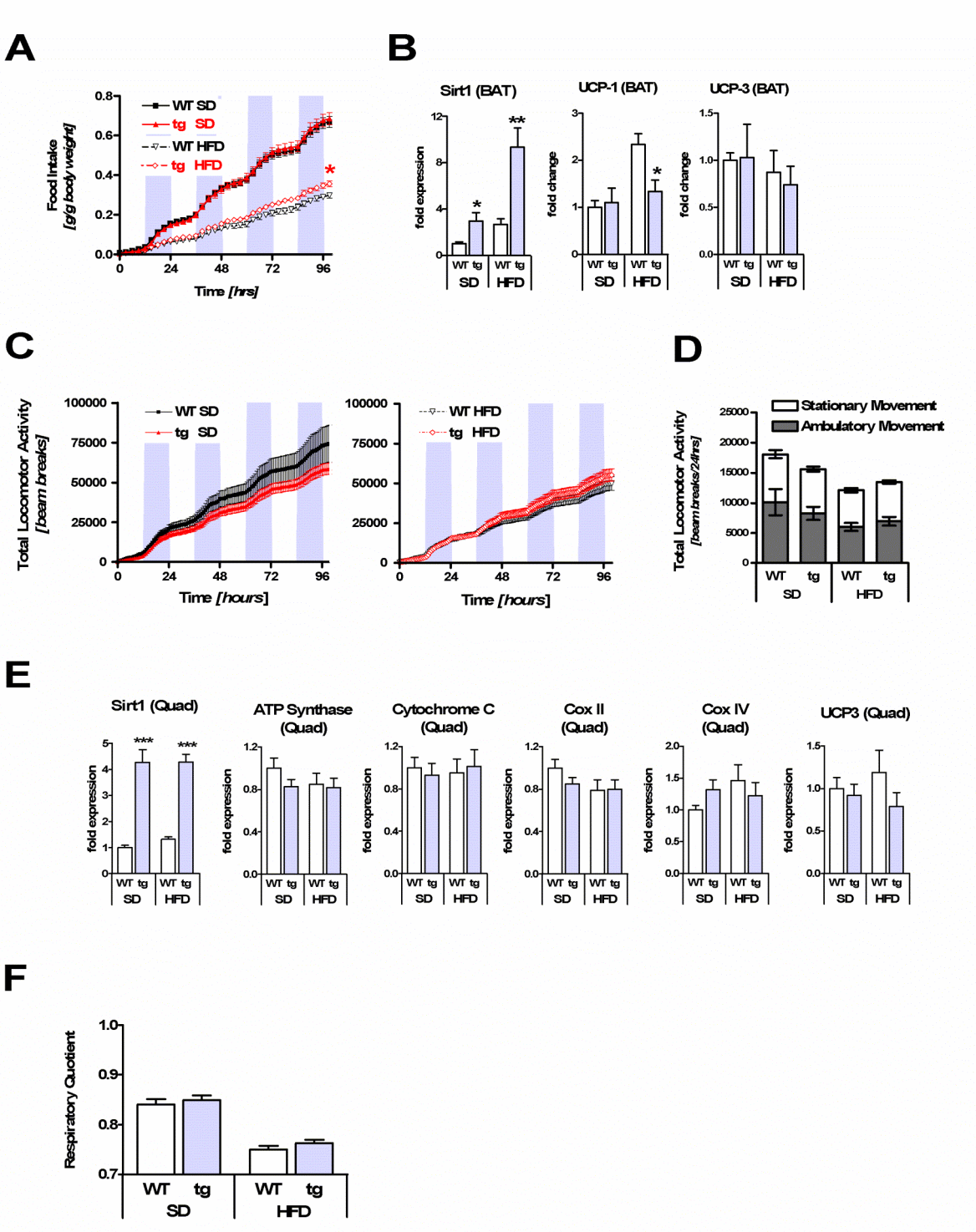


Fig. S4. Energy homeostasis in Sirt1-tg mice. (*A*) Transgenic (tg) Sirt1 overexpressors did not have a different food intake than wild-type (WT) control mice on standard chow diet (SD), but ate significantly more than their WT littermates on a high-fat diet (HFD). (*B*) Real-time PCR analysis of brown adipose tissue (BAT) shows overexpression of Sirt1 in mice fed both SD or HFD (*Left*), a decrease of UCP1 mRNA levels in tg mice on HFD (*Center*), and no changes of UCP3 mRNA levels (*Right*). (*C*) No changes in home-cage locomotor activity were observed between WT or tg mice on either SD (*Left*) or HFD (*Right*). (*D*) Further dissection of locomotor activity revealed no changes in stationary, or ambulatory movements, between WT and tg mice on either diet. (*E*) Real-time PCR of the quadriceps muscle showed no differences in the expression of UCP3, a marker for mitochondrial activity, or in Cyt C, Cox II, Cox IVb, or ATP synthase, all members of the mitochondrial respiratory chain. (*F*) Similar respiratory quotient of WT and tg mice on either diet implying no differences in fuel preference nor in the type of calories burned. $n = 7–8$ per group; means ± SEM; *, $P < 0.05$; ***, $P < 0.001$.

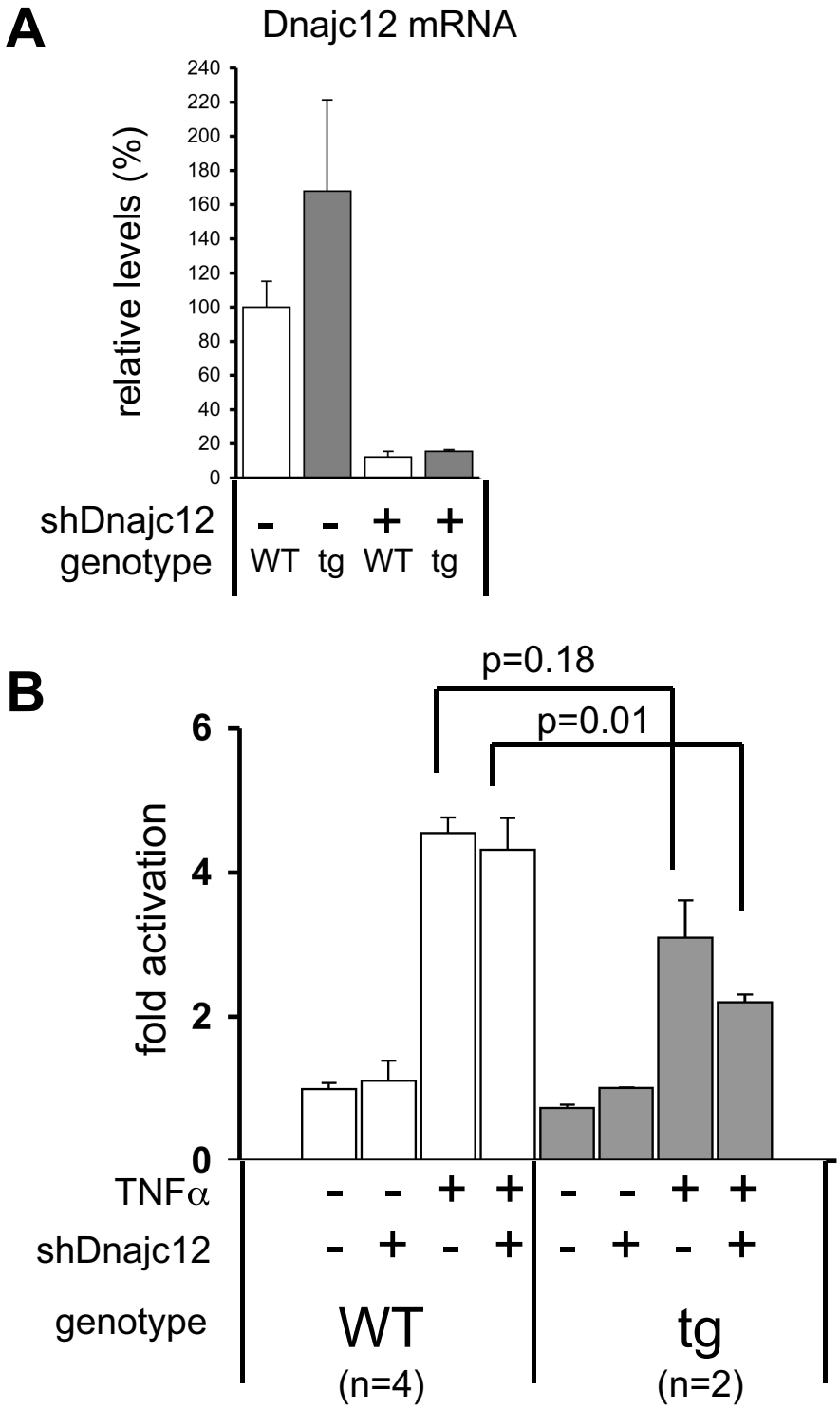


Fig. S5. Lack of involvement of Dnajc12 in the lower NFκB activity of Sirt1-tg cells. (*A*) Levels of Dnajc12 in wt and tg Sirt1 MEFs in the absence or presence of an shRNA against Dnajc12. The shRNA against Dnajc12 (5′-gttgtagtttatttcaata-3′) was designed by using the ''iRNAi'' software and it was cloned into pRetro-Super vector (shDnacj12). Its activity was confirmed by retroviral infection of primary MEFs ($n = 4$ for each genotype and condition) with the empty vector or with the shDnacj12 vector. The primers used for RT-PCR detection of Dnacj12 were: Dnajc12-F 5′-tactacgccttgctgggatg-3′; Dnajc12-R 5′-agttctcaggatgcttgtcgg-3′. (*B*) Luciferase reporter gene assay of WT and tg mouse embryonic fibroblasts after transfection with a pNFκB-Luc reporter vector and stimulation with 10 ng/ml TNFα in the presence of a control shRNA (shGFP, indicated as ''−'' in the figure) or of a shRNA against Dnacj12. Luminescence was measured 6 h after TNFα incubation. Sirt1-tg cells showed a lower NFκB activity regardless of the absence or presence of shDnacj12. Similar results were obtained when shDnajc12 was introduced by retroviral infection (data not shown).

Table S1. Plasma analyses

	SD				HFD				
	WT		tg		WT		tg		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Units
Fast. Glucose 8 wks	134.0	± 5.6	125.0	± 6.0	146.0	± 8.0	142.0	± 8.7	mg/dL
Fast. Glucose 18 wks	115.6	± 3.8	112.0	± 4.0	125.9	± 9.4	135.0	± 7.6	mg/dL
Ad lib Glucose 18 wks	140.8	± 8.7	140.6	± 9.0	145.5	± 6.9	140.1	± 6.4	mg/dL
Fast. Insulin 8 wks	76.9	± 6.2	92.3	± 8.7	161.8	± 21.7	141.2	± 13.8	pg/ml
Fast. Insulin 18 wks	56.0	± 5.4	68.8	± 1.7	115.8	± 11.2	149.4	± 47.4	pg/ml
Fast. Cholesterol 8 wks	58.8	± 1.3	61.7	± 3.8	88.1	± 6.0	85.4	± 5.8	mg/dL
Fast. Cholesterol 18 wks	124.2	± 6.2	125.6	± 5.2	186.8	± 16.6	176.1	± 13.3	mg/dL
Fast. Triglycerides 8 wks	59.7	± 4.0	66.7	± 3.8	97.6	± 9.5	80.9	± 10.6	mg/dL
Fast. Triglycerides 18 wks	53.6	± 3.5	68.2	± 4.8*	73.7	± 5.8	74.3	± 6.7	mg/dL
Ad lib FFA 18 wks	0.141	± 0.030	0.152	± 0.043	0.115	± 0.027	0.132	± 0.033	mM
Fast. FFA 18 wks	0.516	± 0.092	0.497	± 0.041	0.379	± 0.033	0.377	± 0.046	mM
Ad lib Leptin 20 wks	5.44	± 0.77	5.42	± 1.12	68.2	± 10.0	49.4	± 11.1	ng/ml
Ad lib Adiponectin 20 wks	4.70	± 0.13	4.61	± 0.22	5.35	± 0.25	5.24	± 0.34	μg/ml

FFA, free fatty acids; means ± SEM, $n = 7–8$, *, $P < 0.05$

Table S2. Sequences of primers

Sirt1 probe A:	Sirt1-A-F 5′-tgacgacttcgacgacgacga-3′
	Sirt1-A-R 5′-tagggcaccgaggaactacctgat-3′
Sirt1 probe B:	Sirt1-B-F 5′-accttggagcaggttgcaggaatcca-3′
	Sirt1-B-R 5′-gctaaagttcccaatgctggtggagc-3′
Sirt1 probe C:	Sirt1-C-F 5′-tccaaacaggcccctgagactaat-3′
	Sirt1-C-R 5′-aacaggcaacagtgtactttctgg-3′
Igf1 probe:	Igf1-F 5′-acacccttcctaacacttg-3′
	Igf1-R 5′-ttaggctccaggctttcgtt-3′
Gapdh probe:	Gapdh-F 5′-tcttcttgtgcagtgccagc-3′
	Gapdh-R 5′-cagtagactccacgacatac-3′
L32 RT-PCR:	L32-F 5′-gccaggagacgacaaaaat-3′
	L32-R 5′aatcctcttgccctgatcc-3′
Sirt1 RT-PCR:	Sirt1-F 5′-cccttctcagtctgctccac-3′
	Sirt1-R 5′-ctccacgaacagcttcacaa-3′
UCP1 RT-PCR:	UCP1-F 5′-gggcccttgtaaacaacaaa-3′
	UCP1-R 5′-gtcggtccttccttggtgta-3′
UCP3 RT-PCR:	UCP3-F 5′-gtctgcctcatcagggtgtt-3′
	UCP3-R 5′-cctggtccttaccatgcagt-3′
CoxII RT-PCR:	CoxII-F 5′-acgaaatcaacaaccccgta-3′
	CoxII-R 5′-ggcagaacgactcggttatc-3′
CoxIVb RT-PCR:	CoxIVb-F 5′-agatgaaccatcgctccaac-3′
	CoxIVb-R 5′-atggggttgctcttcatgtc-3′
CytC RT-PCR:	CytC-F 5′-ccaaatctccacggtctgtt-3′
	CytC-R 5′-gtctgccctttctcccttct-3′
ATP Synt. RT-PCR:	ATP Synthase F1b-F 5′-gagggattaccacccatcct-3′
	ATP Synthase F1b-R 5′-catgattctgcccaaggtct-3′
SREBP1c RT-PCR:	SREBP1c-F 5′-tagagcatatcccccaggtg-3′
	SREBP1c-R 5′-ggtacgggccacaagaagta-3′
IL6 RT-PCR:	IL6-F 5′-agttgccttcttgggactga-3′
	IL6-R 5′-tccacgatttcccagagaac-3′
TNFa RT-PCR:	TNFa-F 5′-cgtcagccgatttgctatct-3′
	TNFa-R 5′-cggactccgcaaagtctaag-3′
Nrf1 RT-PCR:	Nrf1-F 5′-caacagggaagaaacggaaa-3′
	Nrf1-R 5′-gcaccacattctccaaaggt-3′
MnSOD RT-PCR:	MnSOD-F 5′-ccgaggagaagtaccacgag-3′
	MnSOD-R 5′-gcttgatagcctccagcaac-3′